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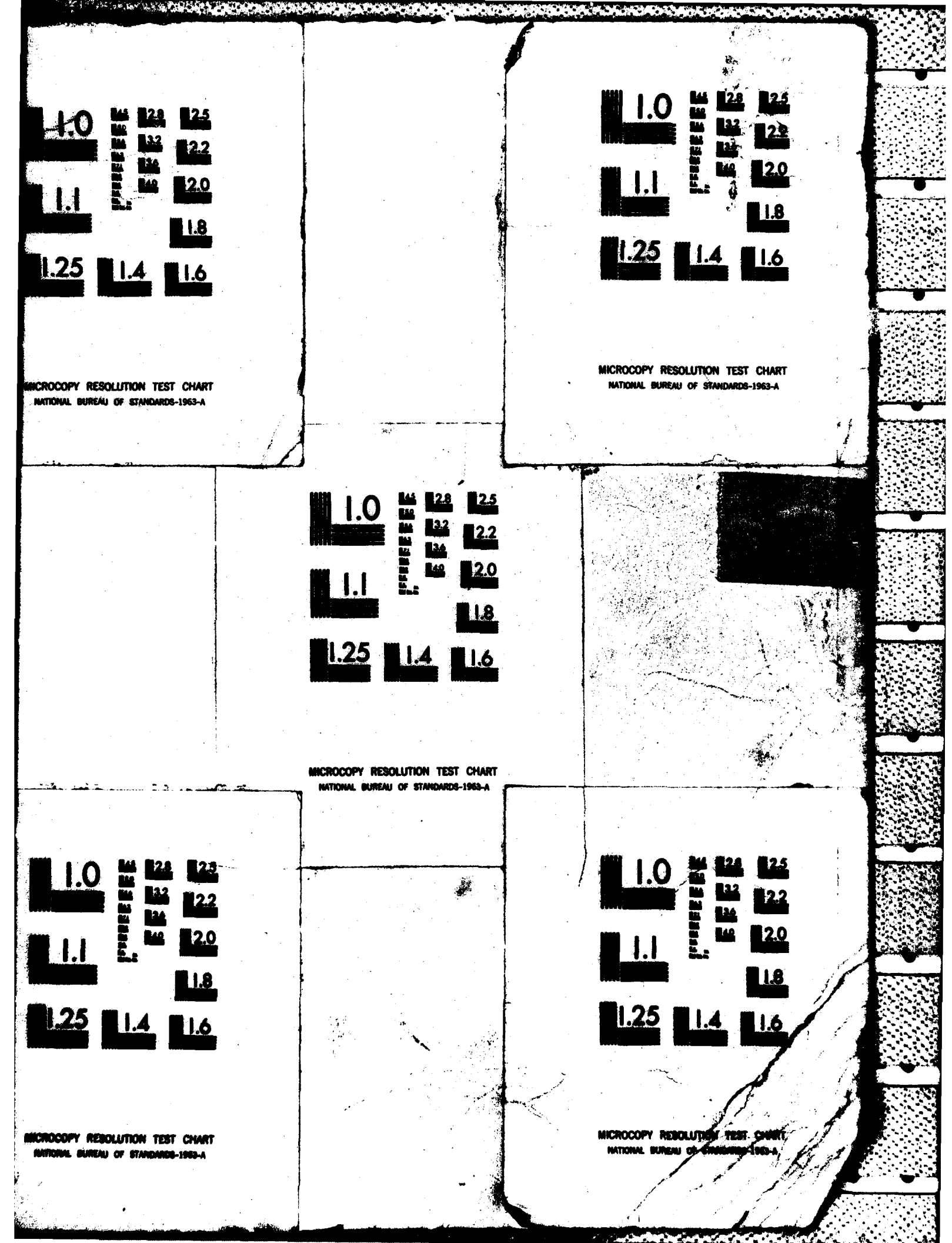
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OFFICE OF NAVAL RESEARCH

Contract No. ONR N00014-76-C-0053

Task No. NR 204-002

FINAL REPORT

16 July 1975-31 Dec 1981

Penetration Enzymes of Schistosome Cercariae

by

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SUMMARY

In view of the facts that schistosomiasis is one of man's most important infectious tropical diseases and that our present capabilities for prevention, control and safe treatment are poor, it was the intent of this contract to work toward prophylaxic by providing basic information about the mechanisms of infection by cercariae. Ingress of cercariae into skin is accompanied by, and is almost certainly dependent upon, emission of secretions from the acetabular glands. To understand the means by which cercariae enter skin, destroy tissue integrity and migrate into blood vessels, the role of the enzymes secreted into skin was studied: simple reproducible means for collecting the secreted enzymes in vitro were devised; pattern of enzyme activity recorded; relationship of enzyme activity to cercarial infectivity established; conditions which influence the activity defined; and the postpenetration cercaria-to-schistosomule transformation described.

Results are recorded under the following headings: (1) cercarial secretions and enzymes involved in penetration and migration in skin; (2) cercarial penetration mechanisms; (3) changes in cercariae transforming to schistosomules; (4) differences in intraspecific geographical strains of *Schistosoma mansoni*; and (5) snail-parasite relationships.

(1) *Cercarial Enzymes Involved in Penetration and Migration of Schistosomes in Skin.* This research was planned against a background of our earlier findings, as follows. (1) Skin surface lipid stimulates schistosome cercariae to penetrate. (2) During penetration, cercariae secrete the contents of their acetabular glands. (3) Skin surface lipid can be used to stimulate cercarial secretion which can be collected in vitro. (4) Since postacetabular gland mucus is not water-soluble, it is the preacetabular gland, water-soluble, enzyme-containing moiety of the secretion which is collected. (5) The active penetration-stimulating fraction of skin surface lipid is the unsaturated polar free fatty acid fraction which must contain the penetration stimulus.

This aspect of the program had . objectives. Objective 1 was to find a substance other than skin surface lipid (SSL) for use in vitro to stimulate quantitatively the emission of preacetabular penetration secretion. Harvests of penetration secretion with SSL were not quantitative. Therefore the free fatty acid fractions of SSL were tested as secretion stimulants. Linolenic and linoleic acids were the most active fractions, in that order, as assayed by enzymatic action on an Azocoll-dye substrate. Activity was directly proportional to the number of cercariae stimulated to secrete. Linolenic acid was therefore substituted for SSL to provide controlled quantitative secretion collections.

As observed microscopically, the cercarial response was the same to linolenic acid as to SSL: immediate movement to the lipidized surface, attempts of cercariae to penetrate, and secretion from acetabular glands. Enzyme activity against the test substrate, dye-coupled Azocoll, was also similar using the two stimulants. (Annual Reports #1, 2, 3; Publication #6)

Objective 2. The optimal concentration of linolenic acid was established. It was 7 to 20 ug per cm^2 of lipidized surface area. Above 70 ug, secretion harvests diminished, probably because of overstimulation and death of cercariae before secretion was complete.

The selected routinely-used technique for collection of secretion was as follows: 7000 cercariae/ml; 10 ml of cercarial suspension containing 100 ug Gentamicin/ml; suspension depth of 3 mm; dish area of 23.75 cm^2; 20 ug of linolenic acid per cm^2 of dish bottom area; incubation for 45 min over a water bath at 40-41 C; self-washing of dishes; suction filtration to remove the parasites. (Annual Reports #1, 2, 3; Publication #6)

The third objective, to establish a base line for secretion enzyme activity throughout the patency of infection in snails exposed to 8 to 10 or to 1 miracidium, required recording cercarial harvests and enzyme activity on a rigid schedule twice weekly for 4 to 6 weeks or until death of all snails. Results indicated that secretion production assayed day to day by enzyme activity in vitro from cercariae from snails exposed similarly on the same day was fairly consistent throughout the patent period. The overall trend of secretion enzyme activity recorded spectrophotometrically per 1000 cercariae followed that of the number of cercariae emerging per snail per day. Both cercarial harvests and enzyme activity of collected secretions, indicating the level of secretion activity, were lowest during the first third of the patent period of the snail infection, highest during the midthird, and gradually decreased thereafter to death of all the snails. (Annual Reports #1, 2, 3, 4; Publications #8, 9, 12, 13, 15)

Objective 4 was to ascertain the relationship of (1) secretion enzyme activity, (2) cercarial production by snails in terms of the average number of cercariae per day, and (3) cercarial infectivity. Since preacetabular gland secretion is emitted into the deeper skin layers as cercariae move through the stratum corneum, there should be a direct correlation between enzyme activity of the cercarial pool assayed in vitro and infectivity of the cercariae, if the enzyme plays an important role in penetration and has a target substrate in skin.

Experimental data showed no daily correlations among these parameters, but overall trends of the curves for cercarial production, enzyme activity and the capacity of the cercariae to infect were similar: low initially and finally, and higher during the midthird of snail patency. (Annual Reports #1, 2, 3)

(2) *Cercarial Penetration Mechanisms.* Ultrastructure of the Penetration Organs of Cercariae. In line with our objective of understanding the mechanisms of skin penetration, the fine structure of the acetabular glands of cercariae was studied with the electron microscope. This was a continuation of our earlier work on gland fine structure. Calcium is known to be present in high concentration in the preacetabular glands of cercariae. Enzyme-containing penetration secretion comes from these glands. Neither the f[illegible] nor the localization of the calcium was known. We therefore localized it. [illegible]n, apparently in the ionic state was found in cercariae in only one type of preacetabular gland granule, namely, the electron-lucid irregularly-shaped inclusions. The same inclusions in penetrated skin still contained the calcium at least for 1 hr. (Annual Reports #2, 3; Publication #2)

(3) *Comparison with Cercariae of Schistosomules Derived in vivo and in vitro.* Since many immunological studies are done with artificially-induced schistosomules, there is need for an assessment of their status as compared with natural postpenetration schistosomules. Furthermore, in terms of prevention

or control of schistosomiasis, a critical stage in the infective process needing elucidation is the transformation in skin of the penetrating cercariae to the migrating schistosomule.

Using cercariae and true in vivo schistosomules for comparison, the histological, behavioral and cytological changes which occur in schistosomules collected and cultured in vitro by a variety of methods, together with the rate of development, was studied functionally and ultrastructurally with reference to the following differences. Cercariae are water-adapted; tailed; rigid and precise in silhouette; CHR positive (the cercarial glycocalyx as antigen combines with an electrophoretically fast-moving gamma globulin as antibody in immune serum to form an envelope); the acetabular glands are full of secretion as demonstrated histochemically with purpurin and PAS; there is no development in culture; and they cannot be successfully cryopreserved. In contrast, schistosomules are water-intolerant; tail-less; worm-like in appearance and locomotion; CHR negative; have emptied their acetabular glands; develop in culture; and are freezing stable.

At the optical microscope level, unstained schistosomules produced by all methods appeared similar in behavior and morphology to those produced in vivo.

Functionally, approximately 99% of all in vivo schistosomules were water intolerant, dying within 15 min. In vitro schistosomules changed more slowly, water intolerance being complete in all types of organisms by 48 hr. In addition to water intolerance, other assessed parameters also indicated slower transition in vitro with the variously derived organisms ranked in descending order of speed of development as follows: rat skin and rat serum; centrifuge-Vortex; and shear and Omnimix.

Examination of the various types of schistosomules with the electron microscope indicated similar rates of development based on the presence of the glycocalyx; lamination of surface membranes; nuclear condition; and cyton granule migration. All types of derivation provided schistosomules, though some more slowly than others. (Annual Reports #2, 4, 5, 6; Publications #7, 14, 16)

Inhibition of Penetration Enzyme. A different approach to the study of the interrelationships of preacetabular gland secretion with cercarial infectivity was based on an earlier finding that an initial exposure to *S. mansoni* cercariae gave mice partial local protection against subsequent homologous challenge through the same skin area for up to 2 days, but not thereafter. Worm burden reductions were 67% at 1 hr challenge, and 50% at 1 day. Protection was essentially against cercarial penetration which was reduced by 20% at 1 hr challenge, 21% at 5 hr, 17% at 1 day and 12% at 2 days.

We tested the hypothesis that the presence in penetrated skin of preacetabular gland secretion from the initially-penetrating cercariae might be the protecting condition, first by studying its effect on the activity of secretion enzymes collected from cercariae held in it.

Secretion collected in vitro from 5000 or more cercariae over linolenic acid did indeed completely block enzyme activity of secretion collected from cercariae held in it, as tested against an Azocoll substrate. Secretion from 2000 cercariae had less blocking effect, while that from 500 cercariae had no effect.

Considering that blockage of the penetration enzyme might be the protective mechanism referred to in paragraph 1, we tested this in mice with the following result. Exposure of mice to 50 cercariae each in secretion from 5000 cercariae as above resulted in no infections since neither worms nor eggs were present at autopsy of the mice sacrificed 8 weeks postexposure. In contrast, control mice exposed concurrently in water were all heavily infected. This protection of mice exposed to cercariae in secretion solution was obviously not an immunological phenomenon, but a chemical one. Immunological activity of the secretion, however, was considered a possibility and was investigated. (Annual Reports #2, 3)

Immunogenicity of Collected Preacetabular Gland Secretion. Secretion was collected from cercariae in vitro, as stimulated by skin surface lipid, and fractioned by disc gel electrophoresis, molecular sieve column chromatography and isoelectric focussing; and mice were immunized according to various schedules of injections of the secretion in adjuvents: Freund's complete or incomplete or alum. Sera were tested for antibody by passive cutaneous anaphylaxis assay, double gel diffusion and protection experiments.

Preacetabular gland secretion was found to be a heterogeneous mixture of components containing both protein and carbohydrate. At least two of the components had proteolytic activity which may be important in the penetration process. Immunization stimulated production of the immunoglobulins IgE and/or IgG. Neither the cercarial secretion antigen alone nor in concert with adult freeze-thaw antigen was protective as shown by absence of reduction in worm burdens of mice after immunization.

Failure to induce protection in any of these systems probably reflects a failure to induce all necessary types of responses which, acting in concert, afford protection. Immunization with cercarial secretion did induce IgE and/or IgG antibodies, but such were not sufficient in quality and/or quantity to protect mice. (Annual Reports #2, 3, 4; Publications #1, 3, 4, 5, 6, 10, 11)

(4) *Differences in Intraspecific Geographical Strains of Schistosoma mansoni.* Evidence has been accumulating that geographical strains of *S. mansoni* differ in terms of epidemiology, morphology and virulence. Daily cercarial output by two strains was recorded for 10 months: PR 1 schistosomes in M line snails and Nmri schistosomes in Nmri snails. Average number of cercariae per day per snail was about 3 times as high in the Nmri-Nmri association as in the PR 1-M. Whether this characteristic is stable, however, or labile and susceptible to genetic selection is not known.

The capacity for cross protection by attenuated cercariae in different substrains of the Puerto Rican snails was also examined in C57Bl/KsJ and NIH/NMRI (CV) mice. Protection was about the same against homologous and heterologous substrains. (Annual Reports #5, 6; Publication #15)

(5) *Effect of Different Snail Exposure Levels on Parasite Development in Snails.* Snails with infections from individual exposures to 8 to 10, 6 to 8, 5, or 1 miracidium did not differ significantly in either average daily cercarial production or patent or prepatent death rates. This may mean that no matter to how many miracidia a snail is exposed, only 1 or possibly 2 concurrently entering invading miracidia develops into primary sporocysts and produces secondary sporocysts and cercariae.

Further evidence for this hypothesis is that the infectivity rates of snails exposed individually to 5 to 7 miracidia and of group-exposed snails (100 snails to 600 miracidia) are essentially equal. This suggests a snail-controlled limit to the number of infecting larvae which can develop. (Annual Reports #2, 4, 5; Publication #15 and one in preparation)

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1. 16 July 1975-15 July 1976

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   C. Base Lines of Day-to-Day Preacetabular Gland Secretion Enzymes

   D. Relationship of Enzyme Activity to Cercarial Infectivity

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   D. Comparison of Schistosomules Derived in vivo and in vitro - Use of Basch 169 Culture Medium for Maintenance of Schistosomules Older than 24 hr

   E. The Cercaria-to-Schistosomule Transformation Stimulus

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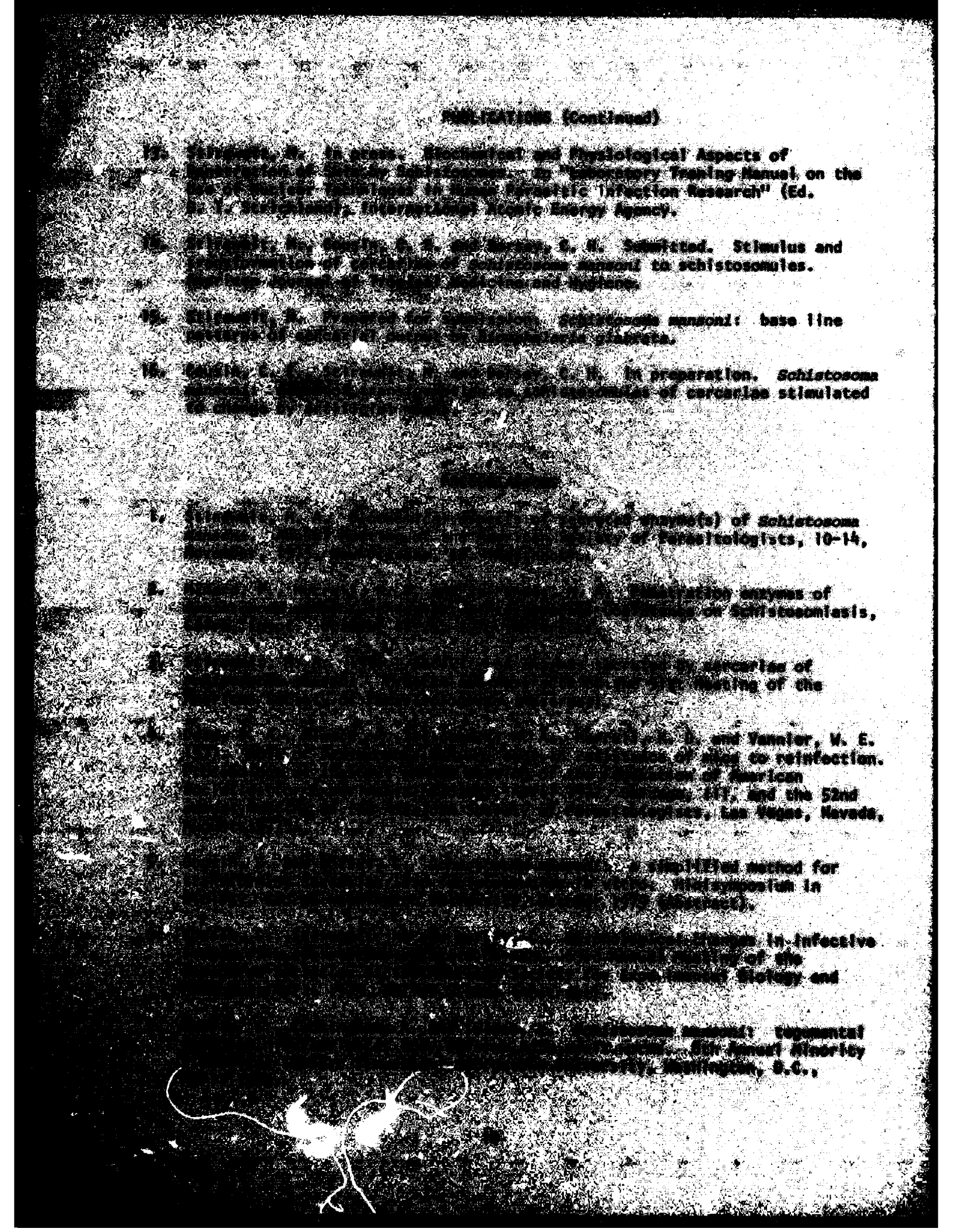

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CONCLUSIONS

1. Certain unsaturated free polar fatty acid components of skin surface lipid, linolenic and linoleic acids, stimulate the cercarial penetration response and the emission of preacetabular penetration secretion which contains proteolytic enzyme(s). These fatty acids can be substituted for skin surface lipid to collect this secretion quantitatively for experimental purposes by procedures devised under this grant.

2. The optimal concentration of linolenic acid in solvent is 7 to 20 ug/cm^2 of surface area to be lipidized.

3. Activity of the secreted enzyme against a dye-coupled collagen substrate is proportional to the number of secreting cercariae. Throughout the patent period of infection of one group of snails, it paralleled the curves for cercarial output (average daily output/snail) and infectivity, in that all 3 parameters were higher during the midthird of patency than during the 1st or 3rd periods.

4. Concentrated collected enzyme inhibits collection of secretion from cercariae held in it, as tested by enzyme activity against dye-coupled collagen. It also inhibits cercarial infectivity. Perhaps the mechanism by which infectivity is reduced is blockage of the penetration enzyme.

5. Immunogenicity of the preacetabular gland secretion is demonstrable by the induction in mice of IgE and/or IgG antibodies but not by protection against a challenge percutaneous infection.

6. Cercariae have unexpectedly high concentrations of calcium. As seen with the electron microscope, the calcium is apparently in the ionic state and is concentrated in the electron-lucid, irregularly-shaped inclusions in the proteolytic-enzyme-containing preacetabular glands of cercariae.

7. Snails of a single strain exposed to 1, 5, 6, or 8 to 10 miracidia each produced the same average number of cercariae per snail per day. Group and individual exposures of snails were equally effective exposure methods.

8. Cercarial output by the various substrain combinations of parasites and snails was substantially different.

9. Intraspecific substrains of *S. mansoni* were cross-protective after immunization with radiation-attenuated cercariae.

10. Rotifers colonized on the snail shells emit a substance which inhibits cercarial emergence, motility and infectivity.

11. Schistosomules can be frozen, stored in liquid nitrogen, thawed and recovered alive. Cercariae cannot, at least by presently known methods.

12. Cercariae transformed to schistosomules by various in vitro techniques instead of penetration of skin are morphologically and functionally similar to those recovered from skin, but the transformation is slower. Those derived by the rat skin and rat serum methods pace in vivo schistosomules more nearly than those derived by centrifuge-Vortex, shear or Omnimix techniques.

MAJOR ACCOMPLISHMENTS

A more comprehensive integrated picture is needed of the invasive process during which schistosome cercariae penetrate vertebrate host skin and migrate in it, concurrently transforming from cercariae to schistosomules. Such picture is pertinent to immunological, biological, biochemical, prophylactic and chemotherapeutic investigations. The following accomplishments contribute to filling this need.

1. First we developed a quantitative technique using linolenic acid for collection in vitro of the preacetabular secretion which contains enzyme(s) which are believed to facilitate some phase of the parasite's penetration into skin. This collection capability opens the way for investigation of the conditions which influence enzyme activity, which is presumably a measure of its concentration, as well as of the enzyme's immunogenicity and possible inhibition, and identification of the target component of skin. It has provided evidence that the level of enzyme activity of cercarial preacetabular gland secretion parallels cercarial development in the snail host, and appears to be reflected in cercarial capacity to infect vertebrate hosts.

2. Base line variability in enzyme production under our conditions has been defined. Establishment of the expected normal level of secreted cercarial enzyme activity and its relation to cercarial harvests and infectivity contribute to understanding the migratory phase of the invasive process. These findings should provide information contributory to the design of a method of penetration inhibition or to a vaccine.

3. Immunogenicity of the secretion in raising IgE and/or IgG antibodies, but not in producing protection, has been demonstrated. Our interpretation is that the antibodies were not present in sufficient quantity or that some supressor was present.

4. Fluctuations in cercarial production and in exposed snail deaths are dependent on specific controllable laboratory conditions for maintenance of the snails and for cercarial collections. As a result of this finding, variability in these parameters has been reduced. Consequently, any schistosome laboratory which is so motivated can produce large numbers of infective cercariae regularly and predictably.

5. The most efficient exposure level for snails, using Nmri strains of *Schistosoma mansoni* and *Biomphalaria glabrata*, was established for our conditions. It was 5 miracidia per snail. Cercarial output was not increased by raising the exposure level.

6. One of the critical conditions for optimal life cycle maintenance is the elimination of inhibitory effect of rotifer-colonization of snails on cercarial output and motility. The effect has been shown to be mediated by emissions from the rotifers. These emissions are water soluble, heat labile and relatively storage stable. It is possible that chemical analysis of the rotifer factor might provide for rotifer control in the laboratory on one hand and for a field control measure against schistosomes on the other.

7. Intraspecific strains of *S. mansoni* were found to vary in cercarial productivity. PR 1 *S. mansoni* in M line *B. glabrata* provided only about 1/3 as many cercariae/snail/day as Nmri parasites in Nmri snails under the same conditions. This appears, however, to be a labile characteristic of the strain relationship and may be subject to genetic selection.

8. Cross protection by intraspecific strains of *S. mansoni* was demonstrated in mice. Among the strains used for immunization and challenge, there was some variability in the level of protection. Greater protection was afforded mice by a chronic infection than by vaccination with irradiated cercariae.

9. Validation of the schistosomular status of artificially transformed cercariae and delineation of their development are pertinent to vaccine production, since many of these organisms are being used in immunological experiments. Understanding the comparative rates of their development, not only in culture but also in ear and lung tissue in vivo, also provides information necessary for planning attacks on the parasites in these host tissues before maturation of the worms. Insofar as has been studied by biological testing and fine structural comparisons of organisms produced by the various methods in use, those produced artificially are acceptable for experimental use. They underwent the same developmental changes as true schistosomules, albeit more slowly.

10. Cercaria-schistosomule transformational morphological changes have been described in detail under natural conditions. These changes occurred in the parasite surface, tegument, tegumental secretory cells, body cell nuclei and digestive tract.

11. A simpler method of producing schistosomules has been devised. It consists merely of centrifuging cercariae to concentrate them and incubating them for 3 hr at 37 C in an isotonic nontoxic culture medium.

12. An additional criterion for distinguishing schistosomules from cercariae has been shown to be the capacity of schistosomules to recover after storage in liquid nitrogen (-196 C). Cercariae handled similarly die.

13. The stimulus for the cercariae-to-schistosomule transformation common to all techniques was incubation in a warm nonlethal isotonic medium for several hours. Without this incubation, none of the preincubation preparative techniques produced schistosomules. Further understanding of the transformation may be of value in blocking development of the penetrated larvae in skin.

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18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Schistosoma mansoni; *Biomphalaria glabrata*; cercariae; eggs; adult worms; miracidia; snails; penetration enzymes; rotifer inhibition; optimal maintenance conditions.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

With an eye toward prophylaxis by interruption of the invasive process, this phase of the schistosome life cycle was studied including: (1) snail-schistosome relationships with special reference to variability in and optimal production of the invasive cercariae; (2) cercarial penetration mechanisms, particularly activity of the penetration enzymes; and (3) postpenetration development of the invading larvae in skin. Reporting in more detail, our findings included the following. The expected variability

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20. ABSTRACT (Continued)

in cercarial production and secreted penetration enzyme activity of preacetabular gland secretion was established for our schistosome strain under our maintenance conditions. The most efficient exposure level for our snails was set at 5 miracidia each. Schistosome substrain differences were found in cercarial production and cross protective capacity. A quantitative technique for penetration enzyme collection was developed using 7 to 20 ug of linolenic acid per cm^2 of area. Enzyme activity was directly proportional to the number of cercariae secreting and to cercarial infectivity. Above a certain concentration, presence of preacetabular gland secretion in skin inhibited cercarial penetration, for up to 2 days. Beyond this, it was immunogenic in inducing IgE and/or IgG antibodies, but was not protective as used. Having penetrated skin, cercariae transformed to schistosomules within 1 hr. A simple artificial method was found to produce the transformation artificially in vitro: concentration of cercariae by centrifugation and incubation in lactalbumin hydrolysate in Earle's medium at 37 C for 3 hr. Transformation of cercariae by this or various other in vitro methods provided true schistosomules as compared with postpenetration schistosomules, but the conversion was slower. The transformation stimulus was identified as incubation in a warm nonlethal isotonic medium.